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# Tuftelin – aspects of protein and gene structure

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The acidic enamel protein tuftelin has now been cDNA cloned, sequenced and characterized in a number of vertebrate species. Recently, the bovine tuftelin gene structure was elucidated. Cloning of the human tuftelin gene and partial sequencing of a number of exons have also been achieved. Immunologically, the protein has been shown to be conserved throughout 550 million years of vertebrate evolution. The gene has been localized to the long arm of the autosomal chromosome 1. The mapping of the human tuftelin gene to a well-defined cytogenetic region could be important in understanding the etiology of autosomally inherited amelogenesis imperfecta, the most common hereditary disease of enamel. The present paper reviews the primary structure, mRNA/cDNA structure, and gene structure of tuftelin. It describes its immunolocalization at the light microscope level and at the ultrastructural level in both the ameloblast cells and in the extracellular enamel matrix. The timing of tuftelin expression and its possible roles in enamel formation are discussed.

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Enamel, a unique and highly mineralized ectodermal tissue covering vertebrate teeth, is synthesized and secreted by specialized cells of the enamel organ called ameloblasts. The ameloblast cells and the secreted extracellular organic matrix provide the environment for the commencement of enamel mineralization and the growth of elongated mineral crystallites. As enamel further mineralizes and matures, most of the extracellular matrix proteins are degraded and replaced with the mineral ions calcium and phosphate, the enamel finally becoming the hardest and most mineralized tissue in the body.

The extracellular organic matrix of enamel is composed mainly of the abundant *amelogenins* and of the non-amelogenin acidic proteins originally termed *enamelins*. Both amelogenin and enamelin proteins have been postulated to play major roles in the development, structural organization and mineralization of developing enamel. The abundant amelogenins (over 90%) are generally hydrophobic in nature, have a molecular weight of about 30 kDa and below, are rich in proline, histidine, leucine and glutamine, and are degraded and lost during the mineralization process (1). Studies on the inhibition of amelogenin translation, using amelogenin antisense oligonucleotides *in vitro* (2), studies on the inhibition of amelogenin mRNA translation using specific amelogenin ribozymes *in vivo* (3), studies on the involvement of mutation in the X-chromosome amelogenin gene, with the amelogenesis imperfecta inherited enamel diseases (4–8), and recent structural studies on the recombinant mouse amelogenin (9, 10) together strongly suggest that amelogenin is involved in the control of mineral crystal size, morphology and orientation.

The non-amelogenin acidic enamelins, on the other hand, have been suggested by many researchers – on the basis of the timing of their appearance (prior to commencement of mineralization), their localization at the dentin-enamel junction (DEJ), their secondary structure, their acidic nature, and their binding to crystal surfaces – to be involved in the initial mineralization of enamel (see review 1, 11). The major acidic enamel matrix proteins, which have recently been characterized, and at least some of which belong to the group of proteins originally described as enamelins, include the acidic enamel protein *tuftelin*, originally cloned and sequenced in bovine and human by DEUTSCH *et al.* (1, 11–16). Very recently, the gene of bovine tuftelin has been cloned and characterized (17), and the (partial) cDNA sequences of mouse and porcine tuftelin have been reported (18; SIMMER *et al.*, personal communication).

Another acidic enamel matrix protein was recently cloned and sequenced in the rat and mouse simultaneously and independently by two different laboratories, which called it *ameloblastin* (19) and *amelin* (20), respectively. The porcine protein has also been sequenced and called *sheathlin* (21).

The acidic enamel matrix protein originally described as a proline-rich non-amelogenin protein and later as a 32 kDa enamelin protein (the precursors of which were shown to be the 89 kDa and 132 kDa proteins (22)) has recently been cloned and cDNA sequenced (23) and the protein called *enamelin*. It is the largest enamel protein characterized to date. Finally, the acidic enamel matrix *sulfated proteins* (about 65 kDa) have recently been identified by SMITH *et al.* (24) but are yet to be cloned and sequenced.

In recent years, it has become clear that some of the proteins often found in what was originally described as the enamelin-enriched fraction are serum albumin proteins (25, 26). However, it has now been shown unequivocally that albumin is not synthesized by the ameloblast cells in normal enamel (27–29). The presence of serum albumin in developing enamel is probably due to contamination during tissue preparation. This finding does not, however, exclude the possibility of the presence of serum albumin in pathologically forming enamel.

## Primary structure of tuftelin

The first acidic enamel protein to be characterized was tuftelin (12–16, 30). The identity, expression and ultrastructural localization of tuftelin was demonstrated using cDNA cloning and sequencing, Northern blot analysis, amino acid composition, indirect immunohistochemistry, Western blot, and high resolution protein-A gold immunocytochemistry, employing synthetic tuftelin peptide antisera (1, 12, 14–16, 18, 31).

The protein, deduced from cDNA sequence analysis, is acidic, hydrophilic, and rich in glutamic acid and aspartic acid. It contains 389 amino acids and has a calculated peptide molecular mass of 43 814 Da. Computer analysis of the predicted protein (from cDNA structure) (16) (see also Fig. 1) reveals one potential N-glycosylation site at residue 298. In addition, seven potential O-linked glycosylation sites have been predicted according to the method of HANSEN *et al.* (32). These include three threonine O-glycosylation sites (at residues 7, 102 and 304) and four serine O-glycosylation sites (at residues 6, 104, 105 and 362). Post-translational modification of the nascent tuftelin protein, such as glycosylation, could increase the molecular weight predicted from its amino acid sequence or cDNA sequence. Indeed, Western blotting of an enamelin-enriched fraction with synthetic tuftelin peptide antisera revealed the presence of tuftelins with different molecular weights of about 66–58 kDa, 48 kDa, 28 kDa and below. These are typically recognized by affinity-purified polyclonal antibodies against enamelins (11) and have been shown to be synthesized by the ameloblast cells (33). The enamelin proteins have indeed been shown to be glycosylated (34, 35).

The different molecular masses of tuftelin protein identified by the specific tuftelin antibodies might also be due to post-secretory processing of tuftelin protein by specific proteases (not as yet identified) or differential mRNA processing (alternative RNA splicing of the tuftelin mRNA transcript) (see below). In this respect, it is interesting that both post-secretory processing (1) and alternative RNA splicing (36–39) are major mechanisms associated with heterogeneity of amelogenin protein.

Five cysteines have been identified in the tuftelin predicted structure. The presence of cysteines might suggest the existence of up to two disulfide bonds, which are generally important in stabilizing the structure of extracellular proteins.

Seven serine and threonine phosphorylation consensus sites have also been identified. Five of these have casein kinase II (CK2) recognition sequences, two of which have serine phosphorylation sites (SKLD and SPPE at residues 70 and 105, respectively), and three which have threonine phosphorylation sites (TWQD, TALE and TIQE at residues 123, 203 and 270, respectively). The remaining two have protein kinase C consensus sequences: SSK and SLR at residues 69 and 119, respectively (Fig. 1). Phosphorylation of the tuftelin protein could provide sites for the specific chelation of calcium ions and thus could be important in understanding the role of these proteins in enamel mineralization.

In the above context, it is interesting that an EF-hand calcium-binding domain consensus sequence, with mismatch, was identified in the predicted tuftelin protein. It was located towards the N-terminal region of the tuftelin protein (DRD PGD SVH KQE I residues 73–85) (Fig. 1). This consensus sequence has been identified in about 30 proteins, some of which are intracellular, but at least one of which is an extracellular secreted protein, osteonectin, which is a non-collagenous bone protein. A mismatch in the relatively large consensus sequence might indicate a weaker association with Ca ions. This weaker association, in turn, might be an important characteristic of a possible nucleating protein.

Recently, PAINE *et al.* (40) using the yeast 2 hybrid system showed that tuftelin could self-assemble (as can the amelogenin molecule), and

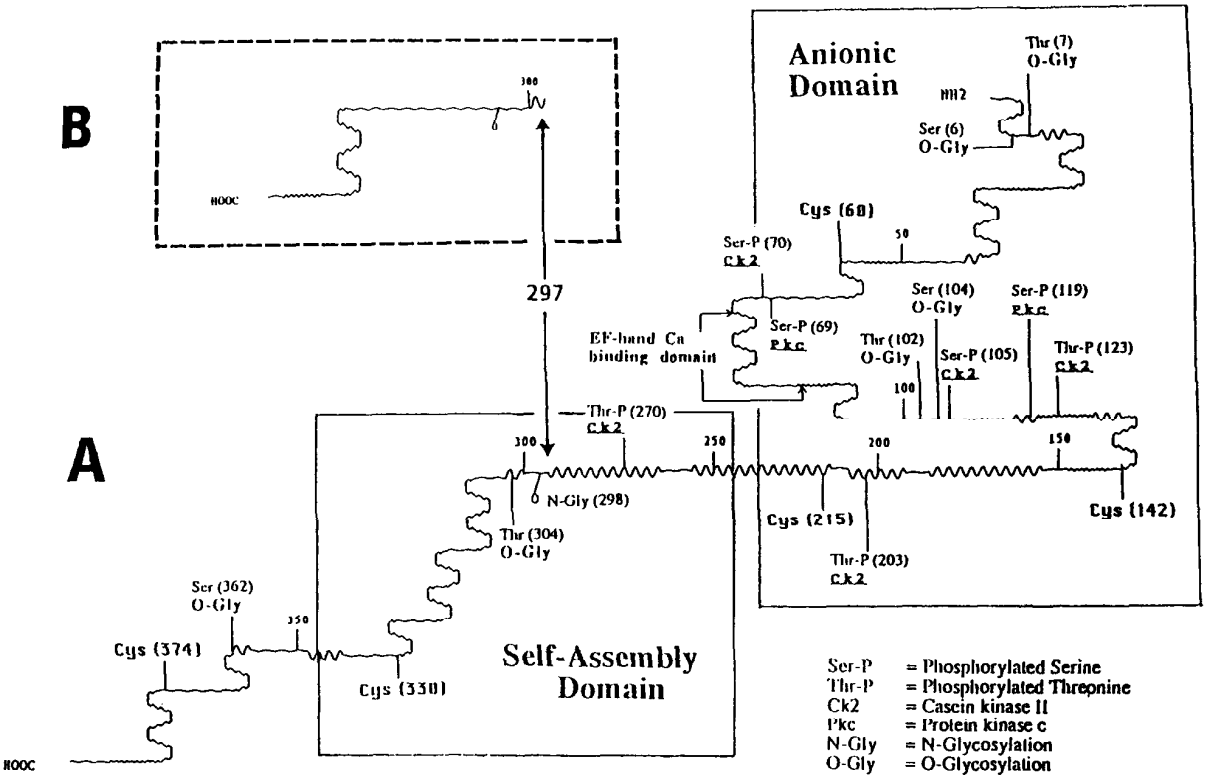


*Fig. 1.* Positions of the deduced tuftelin (a) self-assembly domain (40) towards the C-terminal region, and (b) the anionic domain towards the N-terminal region. The EF-hand calcium binding domain consensus site, the predicted sites for phosphorylation and N-glycosylation sites and the predicted O-glycosylation sites are superimposed on the predicted Chou-Fasman model for protein secondary structure. (A) represents the sequence first deciphered and published (12), for bovine tuftelin. (B) represents the sequence recently found by BASHIR *et al.* (17).

that tuftelin amino acid residues 252–345 adjacent to the C-terminal region contained structurally relevant determinants for this self-assembly. Perhaps this special structural arrangement (Fig. 1) of two major domains, a self-assembly domain at the C-terminal, with a non-self-assembly N-terminal anionic domain (containing seven phosphorylation sites and an EF-hand calcium-binding region) is a structural motif in the role of tuftelin in enamel mineralization.

The tuftelin sequence contains the peptide sequence -Pro-Ser-Pro-Pro-Ala-Gln-. An homologous sequence -Pro-Ser-Ser-X-X-Ala-Gln- is found as part of an N-terminal decapeptide sequence in a 22 kDa unidentified protein band isolated from an enamelin extract (26) (the latter sequence has an extra serine). This may indicate that the published peptide sequence (26) is a tuftelin derivative or fragment.

Very recently, the bovine tuftelin gene was cloned by BASHIR *et al.* (17) and its structure deciphered by DNA sequence analysis and comparison to bovine tuftelin cDNA. Although identical to the sequence of the first 297 amino acids (with the exception of Asp being replaced with Glu at position 190) originally described by DEUTSCH *et al.* (12), because of an additional G found at base position 1097, which caused a frame-shift (see below), the predicted amino acid sequence of the protein at the carboxy terminal region of the protein is changed and a shorter protein is coded for (Fig. 1, forms A and B). The change in the C-terminal of the protein results in a protein containing 338 residues, with a calculated peptide mass of 38630 instead of 389 residues with a calculated mass of 43814 in the previously published sequence (12). The protein contains only three cysteines, compared to five in the published sequence. It does not contain tryptophan but, unlike a previous report by BASHIR *et al.* (17) where no N-glycosylation was found, in a more recent study, BASHIR *et al.* (41) did find a consensus sequence (NFS) for potential N-glycosylation at residues 304–306. It is interesting that the one N-glycosylation sequence found in the originally published tuftelin sequence (12) is in close proximity, at residues 298–300. In both predicted proteins (12, 41), this N-glycosylation consensus site

occurred after residue 297, i.e. immediately after the change of sequence towards the C-terminal (Fig. 1). It is interesting that the predicted structure (Chou-Fasman) at the C-terminal is very similar despite the change in amino acid sequence.

The calculated isoelectric point is 5.85 compared to the 5.2 of the published sequence. Chou-Fasman and Kyte Doolitle analysis indicated that the N-terminal one third of the molecule contains a number of $\beta$ turns, while the middle portion is mainly in an $\alpha$ helix conformation (41). In the original sequence (12), the carboxy region contains more $\beta$ turns and has a shorter hydrophobic region than that found by BASHIR *et al.* (41) (see also Fig. 1). The predicted tuftelin primary structure might even be longer (30, 41).

Recently, the mouse tuftelin cDNA has been cloned and partially sequenced (18). The amino acid sequence of the mouse tuftelin (the cDNA clone of which contained a segment of about 75% of the total sequence published for the bovine) is highly conserved (87%). High conservation was also found between corresponding sequences of bovine and human (14, 41), and bovine and porcine tuftelin (Simmer, personal communication).

## Timing of tuftelin expression

The mouse tuftelin protein was found to be expressed prior to initial enamel mineralization (E17) (18) (see also 12, 15). Expression of tuftelin mRNA, however, was reported to be much earlier, at E13. The difference in sensitivity between Northern blotting and RT-PCR to that of indirect immunohistochemistry might explain the difference in the timing of detection (18). Tuftelin was found to be expressed before any of the other enamel proteins characterized, the sequence of expression being tuftelin-ameloblastin/amelin/sheathlin-amelogenin. Its expression continues throughout the forming stages of amelogenesis. The appearance of the tuftelin protein at very early stages of odontogenesis, and later just before the commencement of enamel mineralization, might indicate that the tuftelin protein is multi-functional (see below).

## Immunolocalization of tuftelin

Indirect immunohistochemical (12) and high resolution protein-A gold immunocytochemistry (15) studies at the light and electron microscope levels, respectively, using synthetic tuftelin peptide antisera (12), showed that the antibodies reacted with the functional ameloblast cells, particularly those in the secretory region (Tomes process) and with the underlying developing extracellular enamel matrix. No apparent reaction was observed with either the odontoblasts or the dentin extracellular matrix. The antibodies also reacted with bovine and human mature enamel tuft proteins radiating from the DEJ towards the enamel surface.

Tuftelin was localized in the ameloblasts in the rough endoplasmic reticulum (RER) and in vesicles and secretory granules in the Tomes process. Double-labeling high resolution protein-A gold immunocytochemistry further indicated that tuftelin and amelogenin proteins do not co-localize in the cell but are found in different vesicles and secretory granules (15). In this context, it is interesting that PAINE *et al.* (40), employing the yeast 2-hybrid system, could not show any interaction between amelogenin and tuftelin. In the extracellular enamel matrix, tuftelin was present mainly at the immediate region of the crystallites. Its distribution throughout the thickness of enamel was not homogeneous: it was more concentrated at the DEJ. This finding agrees with the biochemical and indirect immunohistochemical results of TERMINE *et al.* (34) and DEUTSCH (11) (see also 12).

Using a synthetic tuftelin peptide antisera made to our published bovine tuftelin sequence QSK DTT IQE LKE KIA (amino acid residues 265–279), ZEICHNER-DAVID *et al.* (18, 42) confirmed in the mouse the results observed in bovine tissues (12, 15).

An interesting finding (18) was the immunolocalization of tuftelin at very early stages of amelogenesis (before enamel mineralization, when presecretory ameloblasts, preodontoblasts, and dental papillae mesenchyme cells are present) with the dental papillae mesenchymal (DMP) cells. Further, RT-PCR studies with specific tuftelin primers and mRNA extracted from DMP cell-lines, supported this view. However, *in situ* hybridization studies on tooth bud sections at this very early stage of development are needed to further substantiate this finding.

Immunological studies (1, 12, 13, 15, 42) as well as cDNA sequencing (12, 14, 17, 18, and SIMMER *et al.*, personal communication) revealed cross reactivity and cDNA structural similarity between tuftelin of different species such as human, bovine, rat, mouse, porcine, rabbit and shark, suggesting high conservation of tuftelin structure (or part of it) between species throughout vertebrate evolution. More recent results (VAHADI *et al.* (unpublished) and SATCHELL *et al.* (unpublished)) have shown that tuftelin antisera also reacted with the outer layer of pharyngeal teeth of the Pacific hagfish and with the teeth of the lungfish, respectively (the two constituting very ancient vertebrate species). The conservation of tuftelin throughout 550 million years of evolution suggests that it has an important

biological role in the development and mineraliz ation of enamel.

### Tuftelin mRNA/cDNA structure

The bovine tuftelin cDNA clone (2.7 Kbp) originally described by DEUTSCH *et al.* (12, 30) was isolated by antibody screening from a bovine ameloblast enriched cDNA library in expression vector lambda–Zap2. The DNA sequence was 2674 base pairs long. A methionine codon at base 182 surrounded by a KOZAK (43) consensus sequence (GCC ATT G) for eukaryotic initiation sites is followed by an open reading frame of 389 amino acids extending to a stop codon (TAG) at base 1349. A polyadenylation signal (AAT AAA) begins at position 2637. 180 bases (1–181) from the 5′ and 1322 (1352–2674) bases at the 3′ ends are untranslated sequences. Part of the large 3′ untranslated portion could be involved in the stabilization of the tuftelin mRNA molecule. At the 5′ end, the open reading frame extends to another potential ATG codon (base position 26–28). It is thus possible that the tuftelin primary structure could be even larger (30, 41).

In the bovine tuftelin cDNA sequence described by BASHIR *et al.* (17), the 5′ untranslated portion was 28 bases longer (found by 5′ Race methodology). In addition, as stated above, two differences could be seen in the translatable cDNA open reading frame, one which was a change of C with G, resulting in a change of Asp with Glu at position 190. The second change was an additional G found between base 1069 and 1070, which caused a frame shift, resulting in a change in the amino acid sequence of the C-terminal region (after amino acid 297) (Fig. 1). Repeated DNA sequencing analysis of the bovine tuftelin cDNA clone isolated by DEUTSCH *et al.* (12), by two different sequencing departments in different institutes, have confirmed the original sequence (12) for this bovine tuftelin cDNA clone, i.e., the presence of C and not G, and no additional G between bases 1069 and 1070 (Fig. 2a, b). Sequencing of two other tuftelin cDNA clones isolated from the same library showed identical results. In addition, a polyclonal antibody (LF73) (12) made against a C-terminal region tuftelin synthetic peptide (12), occurring 3′ to position 1069–1070 (where the additional G was present in the new sequence), reacted with major enamelin-enriched fraction proteins (66 kDa and 55 kDa), which typically react with an affinity-purified polyclonal antibody against bovine enamelin (see Fig. 2c) and with tuftelin synthetic peptide antibodies made to the N-terminal and to the middle of the tuftelin molecule (5′ to the position where G is present in the new sequence).

The results above suggest that both forms (A and B) (Fig. 1) of tuftelin might exist but this is yet unclear. Since the B form occurs both in the bovine (data from cDNA and genomic sequences) and in human, there is no doubt that the B form is the more abundant form of tuftelin. BASHIR *et al.* (17, 41) have suggested that the A form, on the other hand, might represent an unusual type of polymorphism. Gene duplication has been suggested but at present no evidence exists for such an explanation.

ZEICHNER-DAVID *et al.* (18) found the proportion of tuftelin mRNA to be 0.1% of that of the amelogenin, and CERNY (personal communication) stated that amelin mRNA was 7% of that of the amelogenin.

It is now clear that alternative splicing of tuftelin mRNA transcripts does occur. Indication for mRNA splicing came from an early study of DEUTSCH *et al.* (30), who reported – using Northern blot analysis – the presence of two mRNA species (2.7 and 1.0 kb, respectively), coding for bovine tuftelin. More recently, studies by BASHIR *et al.* (17) on the gene structure of bovine tuftelin established the presence of four different mRNA transcripts: one which coded for all 13 exons, one mRNA transcript that lacked exon 2, one mRNA transcript which lacked exon 5, and one which lacked exon 6 respectively (Fig. 3). In addition, they found one mRNA transcript that contained a 3′ untranslated segment, which was 240 bases shorter.

### Gene structure, chromosomal localization in man, and amelogenesis imperfecta

Recently, the bovine tuftelin gene was deciphered by BASHIR *et al.* (41). The gene (>28 Kbp) consists of 13 exons, ranging in size from 66 (exon 6) to 1531 bp (exon 13, including the stop codon and the 3′ untranslated sequence). Exon 3 contains the start codon (see also Fig. 4). The exon/intron border sequence matched consensus sequences deduced for a large number of eukaryotic genes. Generally, the border codons are not split.

Employing the bovine cDNA clone (12), DEUTSCH *et al.* (14) first cloned (from a human genomic library contained in a Strategene lambda Fix) and partially sequenced (4 exons have now been sequenced, Fig. 4) the human tuftelin gene. A high homology was observed between the human exon and the corresponding bovine cDNA sequences. Employing fluorescent *in situ* hybridization, we (14) mapped the human tuftelin gene to chromosome 1q 21–31. The mapping of the human tuftelin gene to a well-defined cytogenetic region could be important in understanding the etiology of autosomally inherited amelogenesis imperfecta (AI), the most common hereditary

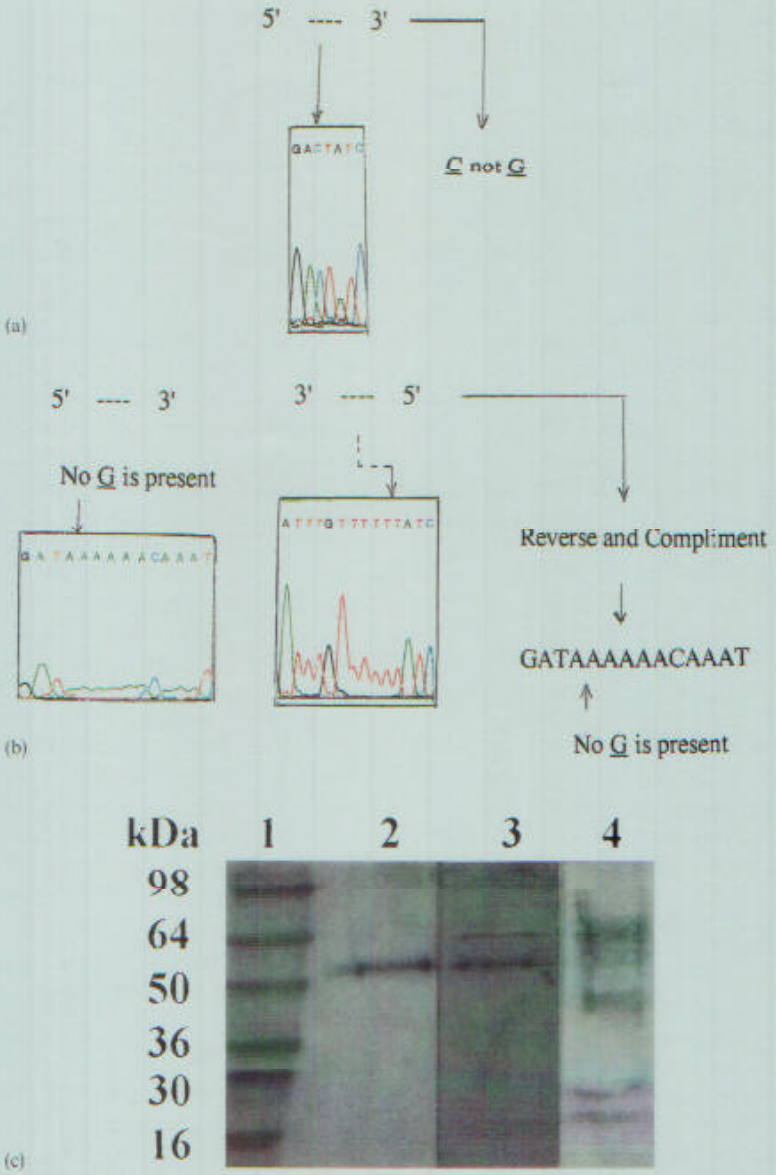


*Fig. 2.* (a) DNA sequence analysis in the 5'-3' direction, confirming the original sequence (12). C is not changed with G in the sequence of our cloned bovine tuftelin cDNA. (b) DNA sequence analysis in both 5'-3' and 3'-5' directions, by sequencing departments of different institutes, confirming the original sequencing data (12) and failing to show in the cDNA bovine tuftelin clone any additional G. (c) Western blot analysis of enamelin-enriched fraction proteins with: (Lane 2): affinity purified antibody against a major bovine enamelin protein. The antibody recognizes a major 55 kDa enamelin protein (normally it also recognizes a 65 kDa and smaller molecular weight proteins (11)); (Lane 3): reaction with LF73 antibody (made against a tuftelin synthetic peptide 73 (12), positioned towards the C-terminal of the protein, the sequence of which is 3' to the position of the additional G in the sequence of BASHIR *et al.* (17, 41)). It recognizes two major enamelin proteins, 65 kDa and 55 kDa, suggesting the presence of a C-terminal protein sequence inferred by the original published sequence (12). (Lane 4): enamelin-enriched fraction proteins separated by electrophoresis, blotted onto nitrocellulose and stained with amido black.

disease of enamel. A mutated form of the tuftelin gene is a good candidate for the cause of at least one form of autosomal AI, because it is expressed in the ameloblasts, is secreted at the early stages of amelogenesis, persists throughout the development and mineralization of enamel, is conserved throughout vertebrate evolution (13, 15), and its gene is localized to an autosomal chromosome (chromosome 1) (14). Attempts are currently being made to study the involvement of the human tuftelin gene in more than 20 families with autosomally linked AI. In this context, it is interesting that recently BÄCKMAN *et al.* (44) mapped the local hypoplastic type of autosomal dominant AI (AIH2) to chromosome 4q. The localiz-

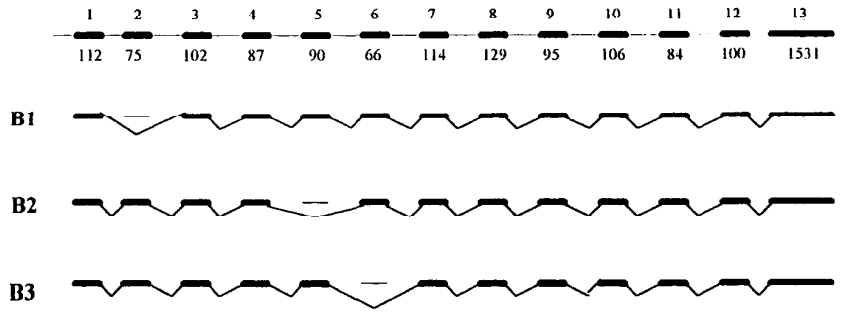


*Fig. 3.* Alternative splicing characterizing the bovine tuftelin gene, as established and recently reported by BASHIR *et al.* (41). Intron-exon structure of the bovine tuftelin gene is presented at the top of the splicing graph. The thin lines represent introns and the thick bars exons. The exons are numbered (1–13) above the bars and the number of bases in each exon is written below. A separate non connecting thin line represents exons removed by splicing.

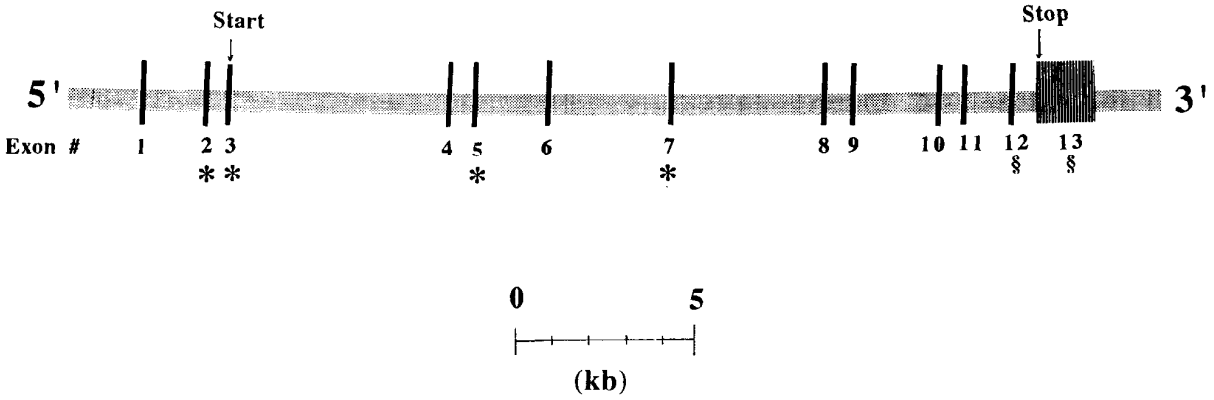


*Fig. 4.* Human tuftelin exons cloned and sequenced to date. These are superimposed on the bovine tuftelin gene structure deciphered and reported by BASHIR *et al.* (41). *-DEUTSCH *et al.* (14), first cloned and partially sequenced the human tuftelin gene and established the DNA sequence of the human exons 2, 3, 5, 7. §Recently, BASHIR *et al.* (41), cloned the 3′ end region of the human tuftelin gene and established the DNA sequence of exon 12 and 13.

ation of the AIH2 locus was refined, and it was further shown that the ameloblastin gene locus was located within the AIH2, suggesting it to be a strong candidate gene for this genetic disease (45).

BASHIR *et al.* (41) have now cloned the 3′ segment of the human tuftelin gene and sequenced exons 12 and 13 (Fig. 4). They found a strong homology in genomic structure and DNA sequence to the bovine gene. Employing fluorescent *in situ* hybridization, they confirmed the results of DEUTSCH *et al.* (14) that the human tuftelin gene is localized at the autosomal chromosome 1q21.

## Tuftelin in enamel development and mineralization

The precise function of tuftelin is still unclear. The acidic nature, some aspects of its 2D structure, its binding to crystal surface, and the timing of its secretion (prior to enamel mineralization) have suggested that tuftelin might be important in the mineral induction of enamel. The presence of relatively high concentrations of tuftelin at the DEJ during early stages of development has suggested to some that tuftelin might be involved with the mineralization of the hypermineralized enamel region adjacent to the DEJ (present in the enamel that forms very early) (46), thereby creating a mineralization front in enamel. This implies that the ameloblasts, together with early forming extracellular enamel matrix proteins, which they secrete at the DEJ, could provide a micro-environment for the commencement of initial enamel mineralization, nucleation, and growth of thin enamel crystallites.

Since tuftelin has been shown to be expressed at very early stages of odontogenesis (bud stage) (18) and amelogenesis (presecretory stage), prior to the commencement of enamel mineralization (11, 12, 15, 18), it may be a multifunctional protein and have other roles in the initial mesenchyme/ectoderm interactions and communication leading to enamel and dentin formation (16, 18).

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